BIOTHERAPEUTICS POTENTIAL OF HUMAN GUT MICROBES OF ROURKELA, ODISHA AGAINST ENTERIC PATHOGENS

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By

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DECLARATION

I hereby declare that the thesis entitled "**Biotherapeutics Potential of Human Gut Microbes of Rourkela, Odisha Against Enteric Pathogens**", submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Rasu Jayabalan, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Rourkela

Date: 11- 5 - 2015 Shilpa Swagatika Tripathy

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CERTIFICATE

This is to certify that the thesis entitled "**BIOTHERAPEUTICS POTENTIAL OF HUMAN GUT MICROBES OF ROURKELA, ODISHA AGAINST ENTERIC PATHOGENS**" which is being submitted by **Ms. Shilpa Swagatika Tripathy,** Roll No. **413ls2054**, for the degree of master of science in Life Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institute for the award of any degree or diploma.

R. Jayabalan. 11/5/15

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Shilpa Swagatika Tripathy

CONTENTS:

LIST OF TABLES:

LIST OF FIGURES:

ABBREVIATIONS:

AAD – Antibiotic-assisted Diarrhoea

CCD - *Clostridium difficile* Colitis

CFU – Colony Forming Unit

DNA – Deoxyribo Nucleic Acid

GIT – Gastro Intestinal Tract

IBD – Inflammatory Bowel Disease

LAB – Lactic Acid Bacteria

MRS – deMan, Rogosa and Sharpe

NB – Nutrient Broth

NB+cys – Nutrient Broth + cystein

SCFA – Short Chain Fatty Acid

SCM – Simulated Colonic Model

ABSTRACT:

Biotherapeutics is a newer approach towards treatment of diseases using living organisms or their products. Probiotics as biotheraputic agents are now the main topic of research that is gaining attention of the scientific researchers around the globe. As the definition given by FAO/WHO probiotics can be defined as live microorganisms that when administrated in adequate amount confers a health benefit on the host. Probiotics when isolated from the human Gastro Intestinal Tract become more specific towards treatment of diseases related to human beings due to population specificity of the probiotics. These gut micro flora play an important role in maintenance of the gut health by conferring certain health benefits and preventing the invasion of the disease causing pathogens. Diseases such as salmonellosis, inflammatory bowel disease, colon cancer, infectious colitis and infection in urinogenital tract are the most common as well as life threatening diseases causing maximum death around the world. So treatment of these diseases can be done using biotherapeutic approaches including population specific gut microbiota as a replacement of traditional medicines and antibiotics. The project work mainly focuses on evaluation of the therapeutic and prophylactic activity of human isolates derived from Rourkela region, Odisha against enteric pathogens.

INTRODUCTION:

Microbes, the diverse group of organisms have occupied their own distinct niche being adapted to the diverse environmental conditions over millions and millions of year. Among all the microorganisms, bacteria in itself is a vast group including all beneficial as well as harmful bacteria. Disease causing bacteria concerned to human beings has compelled us to think bacteria as an organism only with deleterious effect. But certain bacteria also exhibit traits that are beneficial to human beings. Probiotics are the ideal example of bacteria that are friendly to human society.

According to UNFAO/WHO probiotics can be defined as the live microorganisms that when administered in adequate amounts confer a health benefit on the host [FAO/WHO, 2002]. The use of probiotics can be diversified into many different areas such as food, drugs and many other dietary supplements. Specific interaction of probiotics with commensal bacteria in host gut microbiota implies enhanced impact on host. One organism has to satisfy certain criteria to be qualified as a probiotics. These characters can be summarized as follows:

1. Beneficial effects on the host should be efficient.
2. Pathogenicity of the probiotics should be zero.
3. Organisms should be tolerant to the GIT environment (bile and acid tolerant) showing viability for a longer period [Marteau, 2006].
4. Showing higher rate of adherence to the gastrointestinal epithelial cells [Thirabunyanon et al., 2009].

Human has sterile gut microbiota during the time of birth which then gets colonized with microbes from mother and from the environment and develops into a complex ecosystem inhabiting various ranges of microbes [Hooper, 2004]. Most of the microbes of gut ecosystem are gram positive and are with low GC content [Qin et al., 2010]. This microbiome colonization stimulates the growth of the microbes (approximately 1.5Kg) that are 10 times more than that of the host own cells (eukaryotic cells). 400 different species of microbes inhabit the gut microbiota [Rolfe, 1997].The functions that are performed by these microorganisms can be classified into three major categories that are

1. Resistance to the colonization of the exogenous microbes (pathogens) and uncontrollable growth of the indigenous microbes.
2. Enrichment of the innate immune system of host to exhibit quick and infection specific response.

3. Supplements the host system with nutritional complements such as vitamins, enzymes and energy components such as volatile fatty acids [Hayashi et al., 2002].

Life threatening diseases like infectious colitis, colon cancer and inflammatory bowel diseases are more common in current arena. Treatment of these diseases needs a newer approach and replacement of the traditional medicines and antibiotic use. This newer approach involves application of probiotics as a biotherapeutic agent for treatment of several diseases. Biotherapeutics can be defined as the use of living organism or their products which have beneficial effect on the host. The main focus of using probiotics as therapeutic agent is to restore the equilibrium of the gut environment.

REVIEW OF LITERATURE:

NEED OF PROBIOTICS:

Probiotics colonization infers many functions that are beneficial to human beings. Enhancing metabolic activity, protection of the host against potential pathogens and nutritional supplementation are the main functions that a probiotic performs in the human gastro intestinal tract. Nutritional homeostasis is the important phenomena that should be maintained to improve the host gastric condition. Microbial imbalance generally known as dysbiosis leads to the growth of certain subdominant microorganisms known as pathogens. Inflammatory Bowel Disease (IBD) is an example of the dysbiosis mediated gastric disease. Probiotics produce certain metabolites that in association with host enzymes such as cytochrome P450 metabolize the nutrients in the host body [Nicholson and Wilson, 2003]. These metabolized nutrients are then absorbed by the intestinal epithelial cells. Glycans, vitamin K, vitamin B, folate and short chain fatty acids (SCFA) are the metabolites produced by the probiotic microorganisms [Gill et al., 2006]. These microorganisms also have the potential to metabolize substance having carcinogenic effect and other drugs producing either beneficial effect or negative impact [Holzapfel et al., 1998]. Probiotic metabolites are needed to digest certain indigestible fibers. Probiotics also act as antagonistic agent against certain enteric pathogens and prevent certain infectious diseases. Examples of some of these diseases are: salmonellosis, shigellosis, inflammatory bowel disease, lactose intolerance, infection of colon and dysentery etc.

The significance of human gut isolates to be used as probiotics are:

1. Their ability to adhere specifically to the GIT epithelial lining.
2. Recognized as self by human immune system.
3. Prevent pathogen from invading into the host cell.
4. Stimulates immune response in the host on pathogen infection.
5. Tolerant to acid and bile in the GIT.
6. Can be isolated easily from the human.

Probiotics are generally region specific and population specific i.e. the microbiota in the gut of individuals in one region varies from that of the individuals of another region. So probiotics isolated from individuals of a particular region will specifically work for those individuals. Specificity of the probiotics is due to variable environmental conditions, cultural differences and fooding habits of the populations of different regions.

SALMONELLOSIS:

Salmonella is a gram negative invasive pathogenic bacterium that having evolved molecular machineries that allow *Salmonella* to get enter into the host cell, survive intracellularly and replicate within the host cell. *Salmonella* can be classified into various serotypes differing from each other genetically. This genetic difference between the serotypes leads to the development of series of diseases. Salmonellosis is mostly characterized by enteritis. Transmission of *Salmonella* mainly occurs by oral and faecal rout. *Salmonella* that are transmitted from the reptiles cause severe degree of salmonellosis. Salmonellosis may vary from gastroenteritis to septicemia. Symptoms that can be observed during gastroenteritis include nausea, vomiting, cramping abdominal pain, diarrhoea, headache, fever and severe dehydration in case of children and adults. Gastrointestinal symptoms may lead to the development of systemic symptoms which are more severe than that of the symptoms of gastroenteritis. The symptoms of the enteric disease include fever, anorexia, headache, lethargy and constipation. Meningitis and septicaemia are the fatal symptoms of this disease.

ETEROCOCCAL INFECTION:

Enterococcus faecalis is a gram positive, nonsporulating, facultative anaerobe and commensal pathogenic bacterium of GIT causing about 90% of the enterococcal infections.Infections that are caused by *Enterococcus* include endocarditis, sepsis, urinary tract infections and surgical wound infections [Jett et al., 1994]. The most common regions of the body that are affected by *Enterococcus* are urinary tract, endocardium, biliary tract, blood stream, abdomen, burn wounds, central nervous system, soft tissues and periodontal tissues etc. [Gold et al., 1975]. Enterococcal infection includes following steps:

1. Adherence to host tissue
2. Invasion and abscess formation
3. Release of factor for modulation of inflammatory response in the host tissue
4. production of secreted toxic substances

MECHANISM OF ACTION OF PROBIOTICS:

Being an only interface between the environment and host GIT plays an important role of distinguishing pathogens from the non-pathogenic microbes by means of their mode of invasion and presence of organism specific flagella [Borchers et al., 2009]. So colonization of the administrated probiotics in the GIT epithelial cells inhibits the growth of the pathogenic

bacteria by altering their metabolic activities [O'Toole and Cooney, 2008]. Mechanisms that are involved in action of probiotics in human GIT are (figure 1):

1. Inhibition of pathogens by producing antimicrobial substances
2. Competition for nutrition
3. Competitive exclusion
4. Enhancement of epithelial barrier function
5. Immunomodulation

Inhibition of pathogens by producing antimicrobial substances:

Probiotics have the ability of producing potential antimicrobial substances that may range from small molecules to the bioactive peptides and other bacteriocins for inhibition of pathogenic growth. Bacteriocins are the translated product from the bacterial ribosome and can be categorized into 4 classes that are:

A. Class I- small peptides e.g. nisin
B. Class II- small heat stable peptides e.g. pediocin
C. Class III- large heat-labile proteins e.g. helveticin
D. Class IV- complex bacteriocins [Servin, 2004]

Listeria monocytogenes is a pathogen which is inhibited by the production of pediocin by *Pediococcus acidilactici* MM33 from human GIT and other 5 strains of *Pediococcus* spp. by the production of several factors such as hydrogen peroxide, lactic acid, exopolysaccharides and other proteolytic activities [Yuksekdag and Aslim, 2010]. *Lactobacillus reuteri* is a probiotic strain that produces antibacterial substances named Reuterin against certain enteric pathogens [Jones and Versalovic, 2009].

Competition for nutrition:

After consumption of nutrient there may be inter specific competition between the probiotic species and the pathogens leading to significant growth in the probiotics inhibiting the growth of the pathogens. For example *Bifidobacterium adolescentis* S2-1 is the probiotic strain that inhibits the growth of *Porphyromonas gingivalis* by better utilization of vitamin K which is the common growth factor for both the organisms [Hojo et al., 2007].

Competitive exclusion:

Probiotics have the ability of eliminating pathogen infections at the infection site by preventing their adherence to the carbohydrate moieties of the glycoconjugate receptors at the

site of infection on the GIT epithelial cells [Vanderpool et al., 2008]. For example colonization of *Lactobacillus plantarum* 423 in GIT epithelial cells prevents 2 pathogens i.e. *Clostridium sporogenes* and *Enterococcus faecalis* from adhering to the epithelial surface GIT [Ramiah et al., 2008]. *Bacillus subtilis* NC11 in the GIT inhibits the infection caused by *Salmonella enteritidis*.

Enhancement of epithelial barrier function:

Loss of GIT epithelial barrier function and disruption of tight junction enhances the permeability towards pathogens and enterotoxins during pathogenic infections [Guttman et al., 2006]. Presence of probiotics enhances the barrier function by inhibiting pathogenic bacteria to get attached to the epithelial lining [Reiff and Kelly, 2010].

Immunomodulation

Probiotics colonized in the GIT epithelial cells stimulate a cascade of reaction through specific interaction between probiotics and cells of the immune system leading to the development of innate and acquired immune response [Vanderpool et al., 2008]. For instance *Lactobacillus plantarum* has the ability to induce production of pro inflammatory and anti inflammatory substances from mononuclear blood cells.

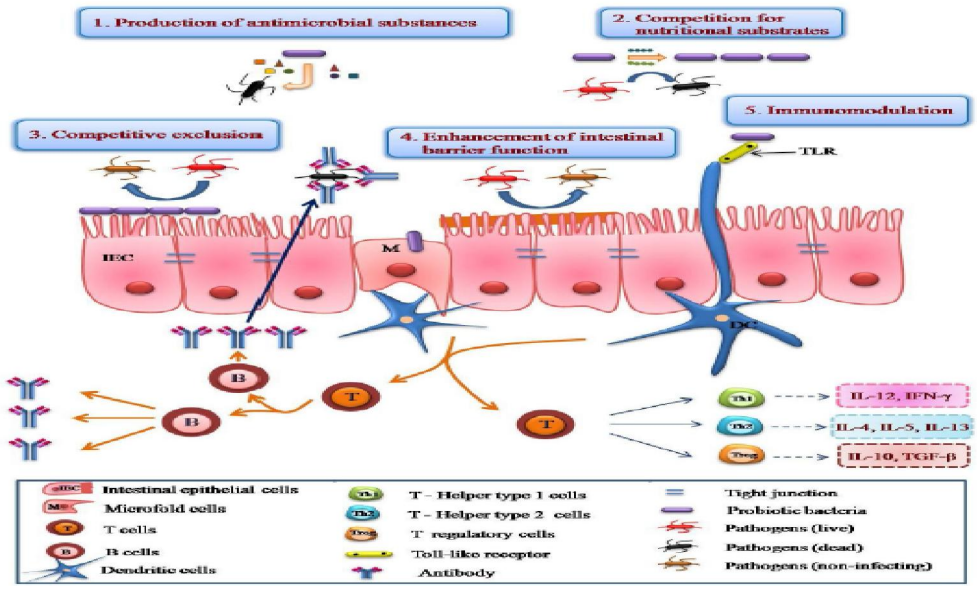

Figure 1: Different modes of action of probiotics [Thirabunyanon et al., 20009]

RECOMENDED DOSAGE OF PROBIOTICS TO BE ADMINISTRATED DAILY:

Different clinical trials have been performed to determine the absolute dose of the probiotics that can be administrated without any adverse effect on the host body. 10^6 CFUs are the

minimal dose of the commercially available probiotics whereas the dose administration may range up to 10^{12} CFUs [Verna and Lucak, 2010]. The result becomes more significant when the dosage of administration increases i.e. $5x10^9$ CFUs is the administration dose for the children whereas adults should be administrated with $10x10^9$ CFUs. The dosage of administration of different probiotic strains concerned with treatment of different diseases is listed in table 1.

Table 1: Recommended dosage of administration of probiotics concerned with different diseases in CFUs

Name of the disease	Probiotics used	Dose to be administrated daily in CFUs	References
Antibiotic-associated diarrhea (AAD)	LABs	10^7 to 10^{11}	Verna and Lucak, 2010
Clostridium difficile colitis (CDC)	*Saccharomyces boulardii*	$2x10^{10}$	McFarland et al., 1994
Irritable Bowel Syndrome (IBS)	*Bifidobacterium infantis* 35624	1×10^8	Kligler and Cohrssen, 2008
Atopic dermatitis	*Lactobacillus rhamnosus* GG	1×10^{10} (adult)	Kligler and Cohrssen, 2008
	Lactobacillus fermentum	$1x10^9$ (children)	Kligler and Cohrssen, 2008
Ulcerative colitis	*Escherichia coli* Nissle	$1x10^{11}$ (induction of remission)	Rembacken et al., 1999
		$5x10^{10}$ (maintenance of remission)	Kruis et al., 1997
	Lactobacillus GG	$18x10^9$	Zocco et al., 2006
	Bifidobacterium longum (along with fructo oligosaccharide)	$2x10^{11}$	Furrie et al., 2005
Crohn's disease	*Lactobacillus* GG	$2x10^9$	Schultz et al., 2004
	Escherichia coli Nissle	$1x10^{10}$	Malchow, 1997

OBJECTIVES:

1. Screening for the therapeutic potential of the human isolates against enteric pathogens.
2. Studies on the prophylactic effect of the human isolates against enteric pathogens.

MATERIALS AND METHODS:

Chemicals used:

Dulbecco's phosphate buffer (10X):

Sodium chloride (NaCl) -80 g/L

Potassium chloride (KCl) -2 g/L

Potassium hydrogen phosphate (KH_2PO_4) -2 g/L

Sodium phosphate ($Na_2PO_4.2H_2O$) -14.4 g/L or Na_2HPO_4 -11 g/L

Calcium chloride ($CaCl_2.2H_2O$) -1.33 g/L (optional)

Magnesium chloride ($MgCl_2.6H_2O$) -1.0 g/L (optional)

Enterococcus agar:

Enzymatic Digest of Casein -15 g/L

Enzymatic Digest of Soybean Meal -5 g/L

Yeast Extract -5 g/L

Dextrose -2 g/L

Dipotassium Phosphate -4 g/L

Sodium Azide -0.4 g/L

2, 3, 5-Triphenyl Tetrazolium Chloride -0.1 g/L

Agar -10 g/L

Final pH: 7.2 ± 0.2 at 25°C

Hektoen Enteric agar:

Enzymatic Digest of Animal -16.5 g/L

Bile Salts Mixture -4.5 g/L

Lactose -12 g/L

Sucrose -12 g/L

Salicin -2 g/L

Sodium Chloride -5 g/L

Sodium Thiosulfate -5 g/L

Ferric Ammonium Citrate -1.5 g/L

Bromthymol Blue -0.065 g/L

Acid Fuchsin -0.1 g/L

Agar -13.5 g/L

Final pH: 7.6 ± 0.2 at 25°C

Lactobacillus MRS Agar: (deMan, Rogosa and Sharpe**)**

Proteose peptone -10.000 g/L

Beef extract -10.000 g/L

Yeast extracts -5.000 g/L

Dextrose -20.000 g/L

Polysorbate -80 1.000 g/L

Ammonium citrate -2.000 g/L

Sodium acetate -5.000 g/L

Magnesium sulphate -0.100 g/L

Manganese sulphate -0.050 g/L

Dipotassium phosphate -2.000 g/L

Agar -12.000 g/L

Final pH (at 25°C) 6.5±0.2

Lactobacillus MRS Broth: (deMan, Rogosa and Sharpe**)**

Enzymatic Digest of Animal Tissue -10 g/L

Beef Extract -10 g/L

Yeast Extract - 5 g/L

Dextrose - 20 g/L

Sodium Acetate - 5 g/L

Polysorbate 80 -1 g/L

Potassium Phosphate -2 g/L

Ammonium Citrate -2 g/L

Magnesium Sulphate - 0.1 g/L

Manganese Sulphate -0.05 g/L

Final pH: 6.5 ± 0.2 at 25°C

Nutrient Broth (NB):

Peptic digest of animal tissue -5.000 g/L

Sodium chloride -5.000 g/L

Beef extract -1.500 g/L

Yeast extracts -1.500 g/L

Final pH (at 25°C) 7.4±0.2

NB+cys media:

Glucose -10 g/L

Calcium chloride ($CaCl_2$) -0.01 g/L

Magnesium sulphate -0.008 g/L

Monobasic potassium phosphate (KH_2PO_4) -0.04 g/L

Dibasic potassium phosphate (K_2HPO_4) -0.04 g/L

Sodium chloride (NaCl) -2.5 g/L

Yeast extracts -10 g/L

Peptone -5 g/L

Cystein HCl -0.5 g/L

Sodium bicarbonate -0.4 g/L

Agar powder -20 g/L

Simulated colonic fluid:

Potassium chloride (KCl) -0.2 g/L

Sodium chloride (NaCl) -8 g/L

Potassium hydrogen phosphate (KH_2PO_4) -0.24 g/L

Sodium hydrogen phosphate (Na_2HPO_4) -1.44 g/L

Proteose peptone -8.3 g/L

Glucose -3.5 g/L

pH -7

Soft agar:

Peptic digest of animal tissue -5.000 g/L

Sodium chloride -5.000 g/L

Beef extract -1.500 g/L

Yeast extracts -1.500 g/L

Agar -8 g/L

Final pH (at 25°C) 7.4±0.2

Pathogens used:

Clinical pathogens were gifted by Dr. Satish Sankar, Sri Sakthi Amma Institute of Biomedical research, Vellore, Tamil Nadu, India.

The list of the pathogens is given bellow;

1. *Salmonella typhi*
2. *Proteus vulgaris*
3. *Proteus mirabilis*
4. *Staphylococcus aureus*
5. *Escherichia coli*
6. *Klebsiella*
7. *Morganella morgani*
8. *MRSA*
9. *Enterococcus faecalis*

Human isolates used:

The human isolates were isolated and screened for various probiotic characteristics from healthy volunteers of Rourkela region, Odisha in collaboration with Dr. Sathpathy, ISPAT General Hospital, Rourkela.

The name of the 5 strains of Human isolates is given bellow;

1. SAE 33 – *Pediococcus spp.,*
2. SAE 27 – *Pediococcus spp.,*
3. SAE 25 – *Lactobacillus fermentum*
4. SAE 13–*Bacillus amyloliquefaciens*
5. SAN 10 – *Bacillus subtillis*

Culturing the pathogens:

NB was prepared and autoclaved. Then 30 ml of broth was transferred to each falcon and pathogen from the pure culture (from colonies of the plates containing differential media) was transferred to the broth. Broth containing pathogen was incubated at 35°C for 12 hrs for broth to become turbid (growth of pathogen).

Culturing the Human isolates:

Lactobacillus MRS Broth (for aerobic human isolates) and NB+cys broth (for anaerobic human isolates) were prepared and autoclaved. 30 ml of broth was poured in each falcon and isolate from the pure culture (from the colonies of the MRS agar plates) was inoculated into the broth. It was incubated at 35°C for 12 hrs for the isolates to grow making the broth turbid.

Agar well diffusion method:

Muller Hinton agar was prepared. Plates and the agar media were autoclaved and 20 ml of the media was poured in each petriplate. After solidification of the media, wells were made on the agar plate using 1ml end cut tips. Pathogens were swabbed on the plate using cotton swabs and allowed to dry. Human isolate culture was centrifuged at 5000 rpm for 10 minutes and 150 µl of cell free supernatant of each isolate was poured in respective labelled wells which were then incubated overnight. Diameter of zone of inhibition was measured and recorded for further observation.

Soft agar overlay method: (spot test)

MRS agar was prepared for the aerobic human isolates (SAE33, SAE27, SAE25 and SAE13) and NB+cys agar was prepared for anaerobic isolates (SAN 10). Four human isolates (i.e. SAE33, SAE27, SAE25 and SAE13) were spotted on each MRS agar plate. SAN10 was spotted on petriplates containing NB+cys media. Volume of the human isolates spotted on the agar plate was 2.5 µl. The plates were then allowed to dry and kept in 35°C for 12 hours for growth of the inoculated human isolates. Pathogens were then inoculated in the soft agar and poured on the top of the human isolates gently and agar was allowed to solidify. After solidification of the soft agar the plates were incubated at 35°C for 24 hours and zone of inhibitions formed by the antagonistic activity of the human isolates against pathogens were observed.

Antibiotic susceptibility test:

MRS agar media for aerobic human isolates and NB+cys media for anaerobic human isolates were prepared and autoclaved. The media were then poured and allowed to solidify. Inoculation of the human isolates was done using a cotton swab. After inoculation of the human isolates, antibiotic discs were placed above the swabbed human isolates and incubated

at 35°C for 24 hr. The diameters of zone of inhibitions produced by the antibiotics against the human isolates were measured.

Co-culture test:

NB, Hektoen Enteric agar and MRS agar were prepared and autoclaved. 100 µl of the pathogen (*Salmonella typhi*) was inoculated into 150 ml of the autoclaved nutrient broth and 100 µl of the pathogen (10^{-1} dilution) (from the150 ml broth) was inoculated onto the Hektoen Enteric plates for initial count of the pathogen. The media with pathogen was incubated in 35°C and after 12 hrs 100 µl of human isolates (SAE33) was inoculated to the pathogen containing media. 100 µl of human isolates (10^{-1}dilution) (from the co-culture media i.e. media containing both pathogen and human isolates) was inoculated on to the MRS agar for initial CFU of the human isolates. The co-culture media was incubated at 35°C and CFU of both pathogen and human isolates was determined at an interval of each 12 hrs by plating them on to Hektoen Enteric (for pathogen) and MRS (for human isolates) agar and counting the colonies.

Aggregation and co-aggregation test:

Human isolates and pathogen samples were centrifuged at 5000 rpm for 10 minutes. O.D of the pathogen and human isolates sample was adjusted to 0.25 (according to Mcfarland standard1) at wave length 600 nm .O.D adjustment was done using phosphate buffer saline. Different experimental set ups were prepared i.e. human isolates control, pathogen control and combination of human isolates and pathogens. The samples were incubated and allowed to co-aggregate with each other (between pathogen and human isolates). O.D of the sample was measured at 600nm at 0^{th}, 2^{nd}, 4^{th} and 20^{th} hours in the microplate reader (96 well plates) and the data were compiled to observe the % of aggregation between the human isolates and pathogens with respect to the control.

Percentage of co-aggregation= [(average of O.D of isolate and pathogen controls - O.D of isolate on probiotics) / average of O.D of isolate and pathogen controls] x 100

Simulated infected colon experiment:

Calibration of nitrogen sparging time:

A solution was prepared containing 2.6g of KOH, 3.3g glucose and 3 drops of methylene blue. Initially the solution was colourless but when saturated with O_2 by sparging the solution appeared blue. Then the solution was sparged with N_2 until the colour disappeared and no blue colour was formed when the tube was shaken. Twice the test tube size twice the head space of the experimental test tube was taken means when the size of the test tube will be doubled the volume of the solution will also be doubled accordingly. The tube was then saturated with O_2 and sparged with N_2 until no colouration was obtained. The time taken for permanent decolouration was noted i.e. 2 minutes.

Inoculation of sample:

Colonic fluid was prepared and autoclaved. 125 µl of human isolates and pathogens having A_{600}=0.25 were added to the simulated colonic model. Inoculation was done using insulin syringe. 40 units in the insulin syringe indicate 1 ml. So for taking 0.125 ml sample should be taken up to 5 units. 3 experimental setups were prepared i.e. probiotic control, combination of human isolates and pathogens and pathogen control. The samples were plated on Hektoene Enteric agar (*Salmonella*), Enterococcos agar (*Enterococci*) and MRS agar (human isolates) at interval of 12 hrs for calculation of CFU.

RESULT AND DISCUSSION:

Objective -1: Screening for the therapeutic potential of the human isolates against enteric pathogens.

Agar well diffusion method:

Table 2: Antimicrobial activity of the isolates against enteric pathogens by agar well diffusion method

Isolates	Inhibition zone radius in mm for the selective enteric indicative pathogens						
	Proteus vulgaris	*Proteus mirabilis*	*Morganella morganii*	*Salmonella typhi*	*Klebsiella spp.*	*Enterococcifaecalis*	*E. coli*
SAE 13	1.5	2.0	1.5	2.0	2.0	-	-
SAE 33	4.0	3.0	3.0	3.0	1.8	-	-
SAE 25	-	-	-	2.0	-	-	-
SAE 27	-	-	-	3.0	-	-	-
SAN 10	-	-	-	-	-	9.0	8.0

- No activity

In agar well diffusion method the cell free supernatant of the isolates were taken which was then poured in the well made on the agar plate. The supernatant then diffused through the agar and zone of inhibitions were observed (table 2). In case of SAE 13 and SAE33, they showed zone of inhibition against *Proteus vulgaris, Proteus mirabilis, Morganella morgani, Salmonella typhi and Klebsiella.* SAE 25 and SAE27 showed inhibition zone only against *Salmonella typhi.* In case of SAN 10 zone of inhibition was only observed against *Enterococci faecalis* and *E. coli*. From the above result it can be concluded that all the isolates except SAN 10 show antagonistic activity against *Salmonella typhi*. As SAE 13 and SAE 33 show maximum antagonistic activity, so these isolates has been considered for further experiments. Presence of zone of inhibition indicates the presence of certain bioactive peptides like bacteriocins, organic acids, hydrogen peroxide that have inhibitory activity against pathogens.

Soft agar overlay method:

Table 3: Antimicrobial activity of the isolates against enteric pathogens by spotting and soft agar overlay method

	Inhibition zone radius in mm for the selective enteric indicative pathogens								
Isolated strains	*Proteus vulgaris*	*Proteus mirabilis*	*Morganella morganii*	*Salmonella typhi*	*Klebsiella spp.*	*Enterococci faecalis*	*E. coli*	*Staphylococcus aureus*	*MRSA*
SAE 13	10	09	>15	15	>15	07	10	08	06
SAE 33	09	08	>15	>15	>15	07	10	11	07
SAE 25	08	07	>15	13	>15	07	08	11	06
SAE 27	08	08	>15	>15	>15	05	12	08	06
SAN 10	-	-	-	-	-	09	08	-	-

- No activity

In case of the soft agar overlay method concentrated form of metabolites produced by the isolates would be made available in the spotted region. This would show a realistic effect happening in the colon where the colon epithelial lining would be colonized by the competing probiotic bacterial species .In colon epithelial lining these isolates may competitively exclude the invasion of pathogens by secreting metabolites which inhibits the growth of pathogens. Thus spot test is used to screen the antagonistic potential of the human isolates with respect to prophylactic and/or therapeutic potential. In this experiment, 4 isolates i.e. SAE 13, SAE 33, SAE 25 and SAE27 showed presence of zones of inhibitions against all the 9 pathogens, these are *Proteus vulgaris, Proteus mirabilis, Morganella morgani, Salmonella typhi, Klebsiella, Enterococci faecalis, E. coli, Staphylococcus aureus and MRSA.*SAN 10 showed zone of inhibition only against 2 pathogens i.e. *Enterococci faecalis* and *E. coli.* Clear zone of inhibitions are observed against *Morganella morgani*, *klebsiella* and *Salmonella typhi.* From table 3it can be inferred that SAE 33has higher antagonism than that of other isolates. So SAE 33 has been taken into consideration for further experiments. Soft agar overlay

method showed higher zone of inhibition than the agar well diffusion because of the higher concentration of the isolates resulting in higher production and diffusion of the substance that shows antagonistic activity.

Co-culture test: (Antagonistic activity of human isolates in NB spiked with *Salmonella* under aerobic condition)

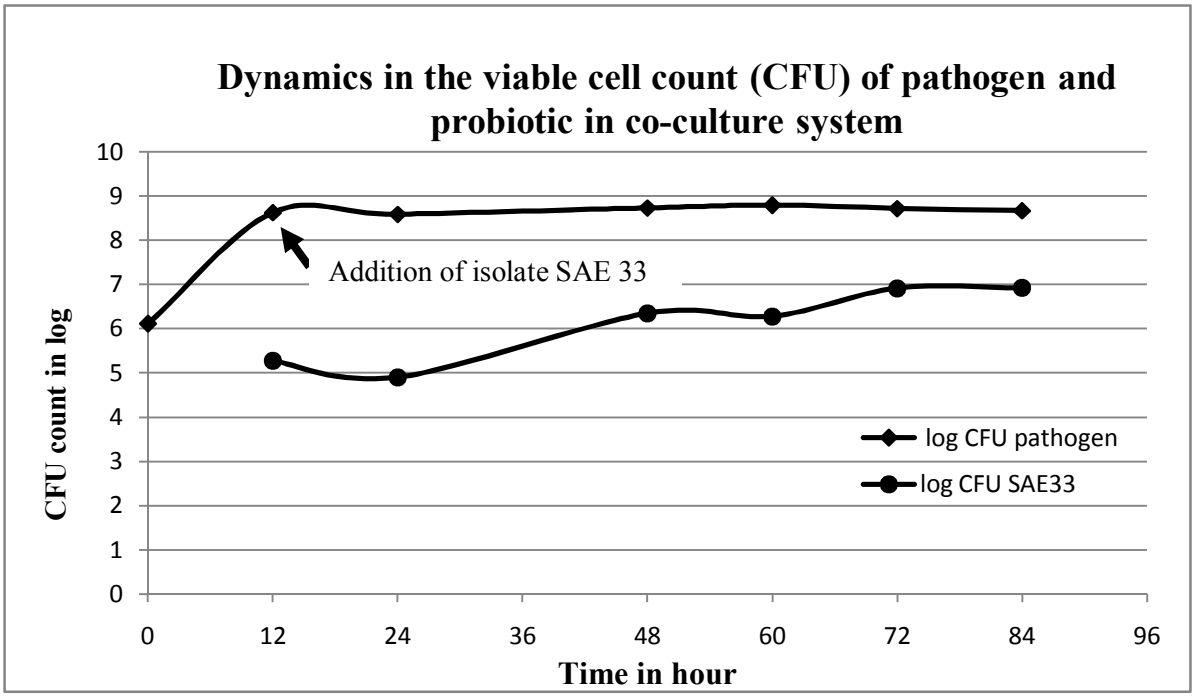

Figure 2: Dynamics in the viable cell count (CFU) of pathogen and human isolates in co-culture system.

In co-culture experiment after inoculation of the pathogen (*Salmonella typhi*) isolate (SAE 33) was added after 12 hrs. So as shown in the graph (figure 2) plotted between time and log of CFU count, *Salmonella typhi* shows logistic growth in first 12 hrs before the addition of the human isolates. After addition of the human isolates the growth of the pathogen was inhibited and a plateau was observed even after 24 hrs of incubation. But there was observable increase in the CFU of the human isolates after 24 hrs. Plateau in the human isolates growth was observed after 72 hrs. From this experiment it can be concluded that in a medium containing human isolates and pathogens, growth of human isolates are higher than the pathogen which then arrested the growth of *Salmonella typhi* by producing antimicrobial substances. This experiment was conducted in aerobic condition as the human isolates were facultative anaerobes the can grow in both aerobic and anaerobic conditions.

Objective -2: Studies on the prophylactic effect of the human isolates against enteric pathogens.

Aggregation and co-aggregation test:

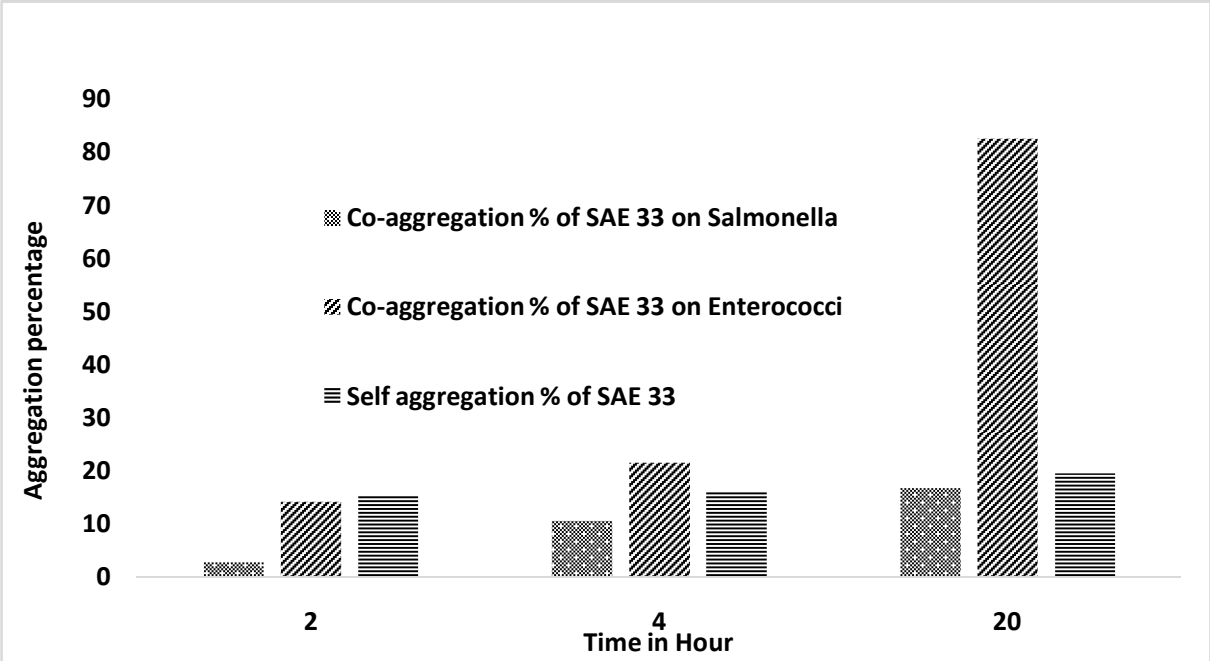

Figure 3: Co-aggregation and self aggregation of human isolate SAE33 on *Salmonella typhi* and *Enterococcus faecalis*

Figure 3 represents 3 experimental set ups i.e. human isolates control (SAE 33), SAE 33 with *Salmonella typhi* and SAE 33 with *Enterococcus faecalis.* After 2 hrs of incubation co-aggregation of SAE 33 with *Salmonella typhi* was least as compared to the co-aggregation of SAE 33 with *Enterococcus faecalis* and self aggregation of SAE 33. But the percentage of aggregation of the both the pathogens with the human isolates increased with increase in the period of incubation. Highest degree of co-aggregation was observed in case of SAE 33 with *Enterococcus faecalis*. The percentage of self aggregation of SAE 33 remains almost constant even after 20 hrs. The co-aggregation of *Salmonnella typhi* with SAE 33 was also notable. As the co-aggregation percentage of *Enterococcus faecalis* with SAE 33 is highest hence SAE 33 showed higher antagonism against *Enterococcus faecalis* than that of *Salmonella typhi*. Result of this reaction can be taken into consideration for inhibition of pathogen by human isolates in a simulated colonic environment.

Simulated infected colon experiment:

Antagonistic activity of SAE 33 in SCF against *Salmonella*:

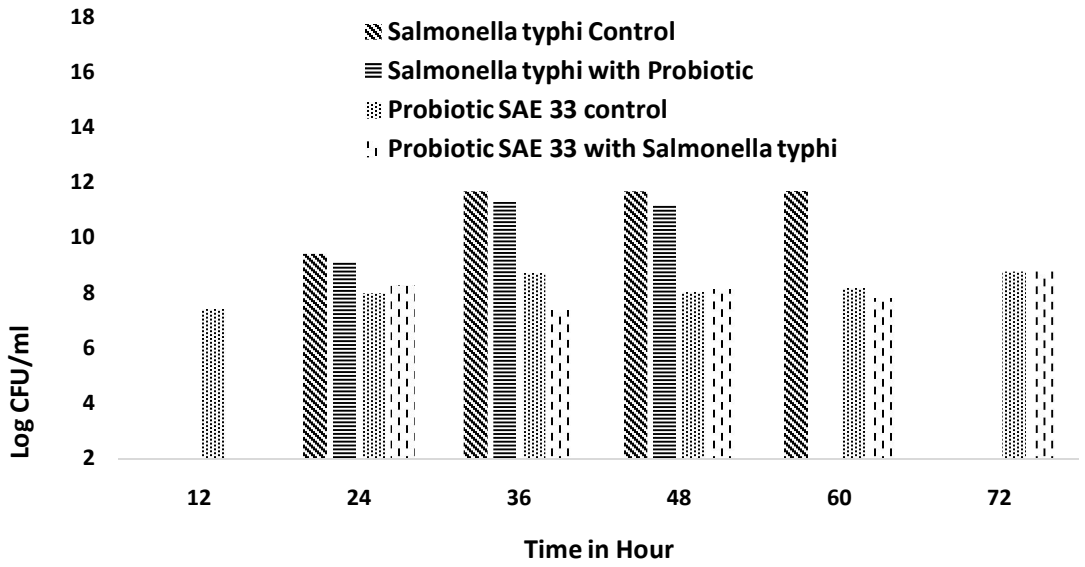

Figure 4: Reduction in viable cell count (CFU) of *Salmonella typhi* in presence of human isolates SAE 33 with respect to control.

Figure 4 represents 4 experimental setups i.e. *Salmonella typhi* control, *Salmonella typhi* with SAE 33, human isolates SAE 33 control and human isolates SAE 33 with *Salmonella typhi*. In first 12 hours only human isolates was incubated in the SCF and there was no pathogen, so the graph represented only the growth of SAE 33 at 12 hrs. In 24 hrs the human isolates count in the tube containing both human isolates and pathogen is higher than that of the human isolates control whereas the pathogen count in the tube containing combination of human isolates and pathogen is lower than that of the pathogen control. In 36 hrs there is a slight decrease in the count of the SAE 33 than that of the control but the pathogen count increases than that of the 24 hrs but the count is still less as compared to the control. The count of *Salmonella typhi* in the tube having pathogen and human isolates completely diminished at 60 hrs whereas growth was still there in the pathogen control. At 72 hrs the human isolates were still detected in growing condition in both the human isolates control and also in human isolates pathogen combination. From this experiment it can be concluded that in anaerobic condition and in an environment similar to human colon the probotic (SAE 33) was showing antagonistic activity against *Salmonella typhi*. As human isolates is active in invitro anaerobic colonic environment, hence its effect will be similar in invivo i.e. inside the human colon.

Antagonistic activity of SAE 33 in SCF against *Enterococci*:

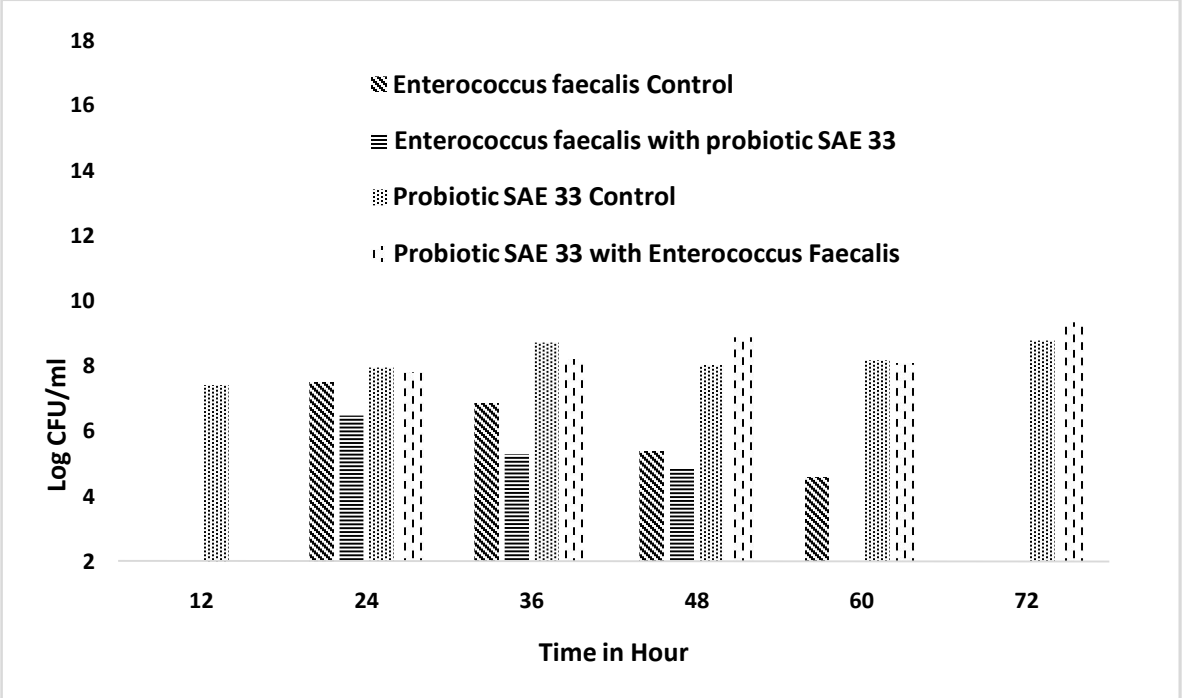

Figure 5: Reduction in the viable cell count (CFU) of *Enterococcus faecalis* in the presence of human isolates SAE 33 with respect to control.

In figure 5 the experimental set up involved human isolates control (SAE 33), pathogen control (*Enterococcus faecalis*) and combination of SAE 33 and *Enterococcus faecalis*. In first 12 hrs there was growth of only human isolates. The growth of the *Enterococcus faecalis* decreased gradually as compared to the pathogen control with increase in time which then ultimately diminished at 60 hrs but growth was still there in the pathogen control. In 72 hrs the count of the pathogen became zero in both the tubes i.e. control and combination of SAE 33 and *Enterococcus faecalis*. In case of human isolates in the tube containing combination of human isolates and pathogen increased gradually with increase in time. This can be inferred from the graph that the survivability of SAE 33 is more than that of *Enterococcus faecalis*. SAE 33 produced certain agents that acted against *Enterococcus faecalis* resulting in reduced count of the pathogen.

Both the above experiments represented that SAE 33 was a potential pathogen having prophylactic activity in the anaerobic colonic environment. This indicated that after colonization in the colon SAE 33 can produce bio chemicals having detrimental effect towards invading pathogens.

Antibiotic susceptibility test:

Table 4: Antibiotic susceptibility of isolated human pathogens against certain antibiotics

Antimicrobial agent	Diameter of zone of inhibition in cm for each isolated strains and interpretation				
	SAE 13	SAE 33	SAE 25	SAE 27	SAN 10
Cepodoxime (CPD) 10 mcg	2.02[c]	1.8[c]	2.6[b]	1.8[c]	2.12[b]
Chloramphenicol (C) 30 mcg	5.2[b]	2.4[b]	5.6[b]	3.4[b]	2.1[b]
Vancomycin (VA) 30 mcg	-[a]	-[a]	-[a]	-[a]	1.8[b]
Streptomycin (S) 10 mcg	2.6[b]	1.2[b]	3.0[b]	2.6[b]	1.4[b]
Rifampicin (RIF) 5 mcg	3.8[b]	-[a]	4.4[b]	3.2[b]	2.0[b]
Levofloxacin (LE) 5 mcg	3.6[b]	1.6[c]	3.6[b]	3.0[b]	1.6[c]
Ceftriaxone (CTR) 30 mcg	2.7[b]	1.4[c]	4.6[b]	3.0[b]	1.6[c]
Clindamycin (CD) 2 mcg	4.6[b]	-[a]	5.6[b]	4.0[b]	2.8[b]
Amoxycillin (AMC) 30 mcg	2.6[b]	1.4[c]	3.6[b]	3.0[b]	1.8[b]
Amikacin (AK) 30 mcg	2.4[b]	1.2[b]	2.8[b]	2.4[b]	2.0[b]
Cefixime (CFM) 5 mcg	3.4[b]	1.6[c]	3.2[b]	2.6[b]	1.2[b]
Tertracycline (TE) 80 mcg	3.0[b]	1.6[b]	4.0[b]	3.2[b]	2.2[b]
Erythromycin (E) 15 mcg	3.0[b]	3.0[b]	3.2[b]	3.1[b]	2.9[b]
Gentamycin (GEN) 10 mcg	1.9[c]	2.2[b]	2.2[b]	2.2[b]	2.1[b]
Penicillin G (P) 10 units	3.5[b]	3.0[b]	3.3[b]	3.5[b]	4.0[b]

Interpretation:[a]resistant, [b]sensitive, [c]Intermediate, according to CLSI
Vancomycinresistence is intrinsic for *Lactobacillus* spp.,
The entirehuman isolates show Methicillin resistance.

A microorganism to be called as human isolates should show susceptibility to all antibiotics. Antibiotic resistance is the intrinsic property of the genomic DNA. If the resistance gene gets incorporated into the plasmid DNA it may get transferred to the pathogen through horizontal gene transfer inducing antibiotic resistance in the pathogens. Induction of antibiotic resistance in pathogen will lead to development of incurable disease in spite of the presence of the antibiotics. In table 4all the human isolates strains show susceptibility toward the antibiotics used except Vancomycin and Methicillin. So these strains can be used as potential human isolates for exhibiting biotherapeutic and prophylactic effect.

CONCLUSION:

The experiments performed above are the standard experiments for evaluation of therapeutic as well as prophylactic activity of the human gut isolates against enteric pathogens. The human isolates used in this experiment show antagonistic activity against the used strains of enteric pathogens. From the co-culture experiment it can be inferred that the presence of human isolate SAE 33 inhibits the growth of *Salmonella typhi* bringing the growth in to a plateau which confirms the therapeutic effect of the human isolate. Aggregation co-aggregation reaction concludes specific binding of the human isolate to the pathogens (*Salmonella typhi* and *Enterococcus faecalis*). The main evidence of the antagonistic activity is obtained by the experiment in simulated infected colonic fluid which concluded that the human isolates can induce antagonistic activity in anaerobic, colonic environment. From the above mentioned experiments it is obtained that SAE 33 is the most potential human isolate having highest antagonistic activity against enteric pathogen. The results of these experiments can be referred for future studies. Future studies about these human isolates may include encapsulation of these isolated strains for administration into the human body. These human isolates can also be incorporated into certain foods such as jelly, yogurt, chocolate and many other forms of food material in appropriate dosage and can be taken in normal diet.

COLOUR FIGURES:

Figure 6: Plate showing antimicrobial activity by agar well diffusion method.

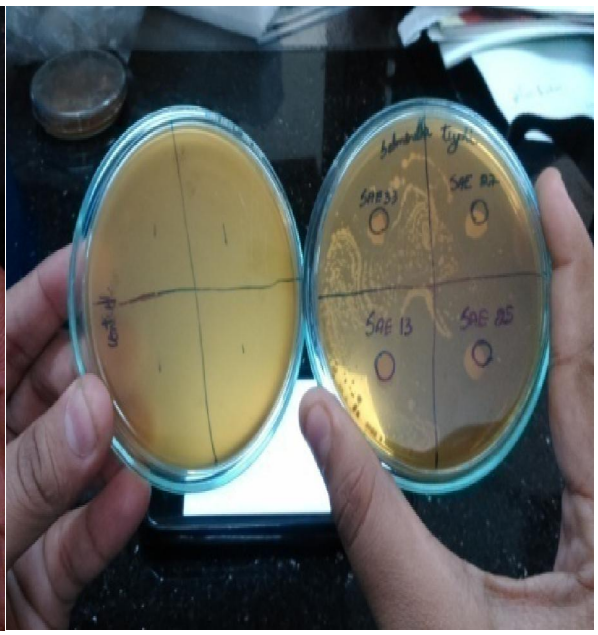

Figure 7: Plates showing antibacterial activity by soft agar over lay method.

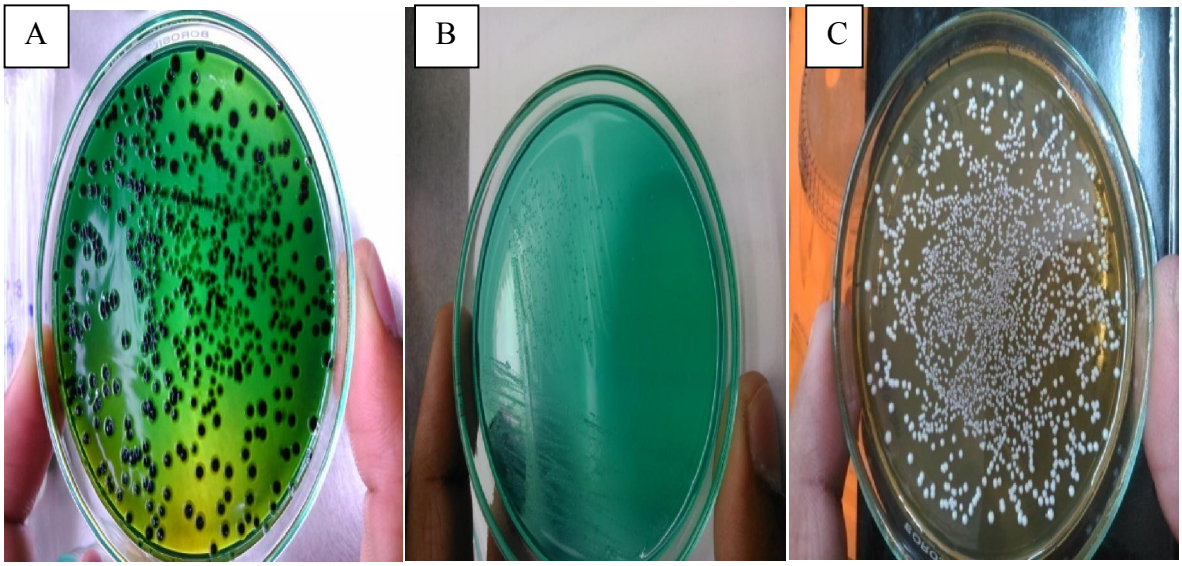

Figure 8: Plates showing growth of isolates and pathogens in differential media
A: Growth of *Salmonella typhi* in Hektoen Enteric agar
B: Growth of *Enterococcus faecalis*in in Enterococci confirmatory agar
C: Growth of isolate in MRS agar

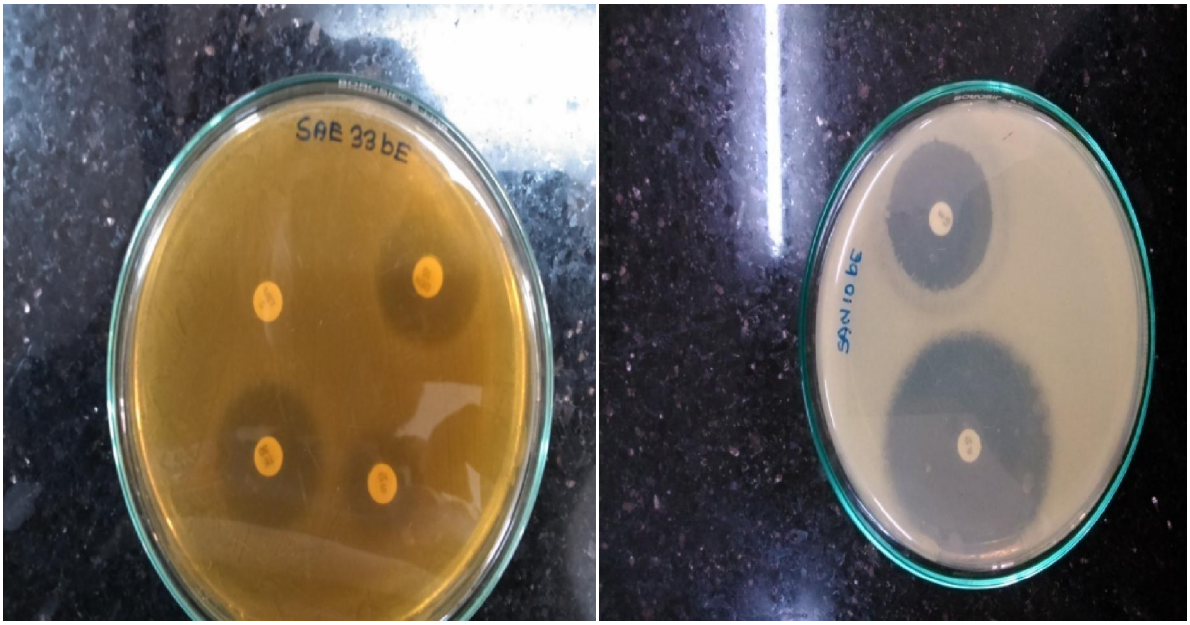

Figure 9: Plates showing antibiotic susceptibility test by disc diffusion method.

Figure 10: Sparging of nitrogen in SCM

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